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Immobilizing Enzymes onto Electrode Arrays by Hydrogel Photolithography to Fabricate Multi-Analyte Electrochemical Biosensors

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Abstract

This paper describes a biomaterial microfabrication approach for interfacing functional biomolecules (enzymes) with electrode arrays. Poly (ethylene glycol) (PEG) hydrogel photopatterning was employed to integrate gold electrode arrays with enzymes - glucose oxidase (GOX) and lactate oxidase (LOX). In this process, PEG diacrylate (DA)-based prepolymer containing enzyme molecules as well as redox species (vinylferrocene) was spin-coated, registered and UV cross-linked on top of an array of gold electrodes. As a result, enzyme-carrying circular hydrogel structures (600 μm diameter) were fabricated on top of 300 μm diameter gold electrodes. Importantly, when used with multiple masks, hydrogel photolithography allowed us to immobilize GOX and LOX molecules on adjacent electrodes within the same electrode array. Cyclic voltammetry and amperometry were used to characterize biosensor electrode arrays. The response of the biosensor array was linear for up to 20 mM glucose with sensitivity of $0.9 \mu\text{A cm}^{-2} \text{mM}^{-1}$ and 10 mM lactate with sensitivity of $1.1 \mu\text{A cm}^{-2} \text{mM}^{-1}$. Importantly, simultaneous detection of glucose and lactate from the same electrode array was demonstrated. A novel strategy for integrating biological and electrical components of a biosensor described in this paper provides the flexibility to spatially resolve and register different biorecognition elements with individual members of a miniature electrode array. Of particular interest to us are future applications of these miniature electrodes for real-time monitoring of metabolite fluxes in the vicinity of living cells.

Introduction

Enzyme-based electrodes represent an important class of biosensors where byproducts of enzymatic breakdown of an analyte are detected electrochemically. (1–5) Of particular relevance are enzyme-based electrodes for detection of glucose and lactate – energy metabolites which are monitored closely in such diseases as diabetes, obesity and cancer. (6–8) Recent emphasis in the development of electrochemical biosensors has been placed on multi-analyte detection and miniaturization. (9–13) The push for miniaturization has been aided by adaption of semiconductor fabrication processes for making and packaging of miniature electrodes. (9,14–18) However, immobilizing distinct biorecognition elements (e.g. enzymes) on different members of an electrode array – a requirement for multi-analyte detection - remains challenging. The enzyme molecules are traditionally immobilized on electrodes using chemical cross-linking, (19) electrodeposition, (20–21) electrostatic interactions (22–24) and membranes

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or carrier matrices.(1–2) These traditional strategies for enzyme immobilization are not well suited for depositing specific enzyme types onto a desired members of an electrode array.

Hydrogels are attractive materials in fabricating electrochemical biosensors because a hydrated gel provides an excellent matrix for encapsulation of functional enzymes.(2,4) A number of studies have used a uniform gel membrane coated on top of the microfabricated electrodes to prevent fouling and enzyme leaching, (16,25–27) while far fewer reports describe integration of patterned gel layer with electrodes.(10,28–29) The previous reports employed acrylamide, hydroxyl ethyl methacrylate (HEMA) or poly vinyl acetate (PVA) for hydrogel/electrode construction.

The goal of the present paper was to utilize poly(ethylene glycol) (PEG) hydrogel photolithography, a micropatterning process reported by us previously,(30) in order to interface enzymes with microfabricated electrodes. PEG is a material of choice for preventing non-specific binding and electrode fouling, (4,31) and has been used extensively for entrapment of functional enzymes.(2,30,32–34) In this paper we sought to leverage excellent properties of this biomaterial in order to interface enzyme-carrying PEG hydrogels with Au electrodes. Our design criteria were: 1) to demonstrate micropatterning of PEG hydrogel structures in registration with microfabricated Au electrodes, 2) to characterize function of glucose oxidase (GOX) and lactate oxidase (LOX) enzymes entrapped within hydrogel microstructures, 3) to develop redox hydrogels that circumvent the dependence of glucose and lactate sensors on diffusible species (e.g. oxygen) and 4) to place of LOX- or GOX-carrying hydrogel microstructures on the adjacent electrodes of an electrode array. The novel fabrication method and a microdevice described here represent an important step towards development of biosensors capable of interfacing with living cells for *in vitro* monitoring cell metabolite fluxes.

MATERIALS AND METHODS

Materials

Poly (ethylene glycol) diacrylate (PEG-DA, MW 575), 2-hydroxy-2methyl-propiophenone (photoinitiator), 99.9% toluene, (EC 1.1.3.4, type II-S from *Aspergillus niger* (18000 U g⁻¹ solid), MgCl₂·6H₂O, D-(+)-glucose, vinylferrocene, and horseradish peroxidase (HRP) were purchased from Sigma (St Louis, MO, USA). Amplex Red was purchased from Invitrogen (Carlsbad, CA). Lactate oxidase (E.C. 1.1.3.2 from *Pediococcus* sp) was obtained from MP biomedical (Solon, OH, USA). Phosphate buffer (PBS) 10 mM was used as a supporting electrolyte for all electrochemistry experiments. Chromium etchant (CR-4S) and gold etchant (Au-5) were from Cyantek Corporation (Fremont, CA). Positive photoresist (AZ 5214-E IR) and its developer solution (AZ300 MIF) were brought from Mays Chemical (Indianapolis, IN). 3-acryloxypropyl trichlorosilane was from Gelest, Inc. (Morrisville, PA). Water used for preparation of aqueous solutions came from a Millipore Direct-Q water purification system (resistivity, 18 MΩ cm⁻²). D-(+)-glucose solutions were allowed to muta-rotate overnight at room temperature before use. Stock solutions were prepared in bi-distilled water or PBS solution and stored in the dark at 4°C.

Equipment

All voltammetric and amperometric experiments were performed using a CH Instruments (CH1910B) Bi-Potentiostat. All experiments were conducted in a three electrode system containing a platinum wire auxiliary electrode, a saturated Ag/AgCl (3 M) reference electrode and enzyme-modified working electrode. The buffer solution was 10 mM PBS with 20 μM MgCl₂. The buffer solution was deoxygenated with N₂ for 10 min before electrochemistry measurements. All electrochemical measurements were performed at room temperature.

Fabrication of Au electrode arrays

The layout of electrode array was designed in AutoCAD, converted into plastic transparencies by CAD Art Services (Portland, OR) and then transferred onto quartz/chrome plates using standard photolithography and wet etching approaches. The design of the Au electrode arrays is shown in Figure 1A.

To fabricate gold electrode arrays, standard (75 mm × 25 mm) glass slides were sputter-coated with 15 nm Cr adhesion layer and 100 nm Au layer by Lance Goddard Associates (Santa Clara, CA). The electrodes were fabricated using traditional photoresist lithography and wet etching processes. Importantly, the photoresist layer was not removed immediately after metal etching but was employed to protect underlying Au regions during the silane modification protocol described below. The etching of Au/chrome layers resulted in an array of five working microelectrodes patterned on a glass slide. Each Au electrode was 300 μm in diameter with 15 μm wide leads and 2 mm × 2 mm square contact pad (see Figure 1A for layout of electrodes). Wires were soldered to contact pads to connect electrode arrays to the potentiostat during electrochemistry experiments.

Integration of Au electrodes with enzyme-carrying hydrogel microstructures

The glass substrates with photoresist-covered Au electrodes were modified using acrylated silane coupling agent following the protocol described by us previously.⁽³⁵⁾ This step was necessary to ensure covalent anchoring of hydrogel structures onto the glass substrates. After silane modification step, substrates were sonicated in acetone for 2 min to remove the photoresist and then placed in an oven for 3 h at 100°C to cross-link the silane layer.

When preparing enzyme electrodes, GOX was dissolved in PBS buffer (pH 6.0) to reach the concentration of 20 mg/mL while LOX was dissolved in PBS buffer (pH 6.5) to concentration of 15 mg/mL. Glutaraldehyde was added to the enzyme solution at 2% v/v to improve enzyme retention and function of the biosensor. In parallel, the prepolymer solution was prepared by adding 2% (v/v) of photoinitiator (2-hydroxy-2-methyl-propiophenone) and 1% (v/v) of vinylferrocene to PEG-diacrylate (DA) (MW 575). Enzyme and prepolymer solution were combined by adding 0.2 mL of the enzyme solution to 0.8 mL of PEG-DA/vinylferrocene. The mixture was stirred for 4 h at 4°C to ensure the homogeneous dispersion of the enzyme molecules.

In the next step, PEG prepolymer solution containing enzyme molecules and redox species was photopolymerized on top of the Au electrodes in a process similar to photolithography (see Figure 1B,C). PEG-based prepolymer solution was spin-coated at 800 rpm for 4 sec onto glass slides containing Au electrode patterns. A photomask was registered with an electrode pattern and then exposed to unfiltered UV light at 65 mW/cm² for 10 sec to convert liquid prepolymer into cross-linked hydrogel. The surfaces were developed in DI water for 3 min to remove unpolymerized PEG precursor solution. Enzyme carrying hydrogel microstructures were made larger than Au electrodes - 600 μm and 300 μm diameter for hydrogel and Au features respectively. This was done to ensure effective anchoring of the hydrogel structures to silanized glass substrate.

The spin-align-expose hydrogel patterning process described above was carried out three times in order to deposit three distinct, co-planar hydrogel layers onto the substrate. The first hydrogel layer contained no enzymes or redox species and was deposited selectively onto the leads, leaving circular electrode and contact pad regions open (see Figure 1B). This step was needed to insulate non-sensing Au regions of the electrode array. In the next step, GOX- and redox species-carrying PEG prepolymer solution was spin-coated and photopolymerized in registration with the desired electrodes of the array. After this step, shown in Figure 1C, select

members of the electrode array contained GOX-carrying hydrogel microstructures. In the final step of fabrication process, LOX-containing redox hydrogels were deposited onto remaining Au electrodes. This multi-step process resulted in construction of an array of miniature Au electrodes with alternating electrodes carrying either glucose- or lactate-sensing enzymes.

To help visualize deposition of different types of hydrogel microstructures on adjacent electrodes, Amplex red-based fluorescence reporter system was utilized. Amplex red is a non-fluorescent molecule that is oxidized into a fluorescent compound during horseradish peroxidase (HRP)-catalyzed breakdown of H_2O_2 . In this experiment, 100 μL Amplex Red/HRP/PEG-DA precursor solution was prepared by mixing 5 μL of 50 mM Amplex Red stock solution with 5 μL HRP (1 mg/mL) and 90 μL PEG precursor solution. Amplex Red/HRP/PEG precursor solution and pure PEG solution were deposited onto adjacent Au electrodes using a multi-step patterning process described above. Hydrogel containing electrodes were immersed in PBS containing 5 mM of H_2O_2 and were observed using upright fluorescence microscope (Nikon Eclipse LV100).

Electrochemical characterization of enzyme-electrodes

Electrodes were tested in a custom-made, Plexiglass electrochemical cell with volume of ~ 1 mL. PBS (pH = 7.0) with 20 μM MgCl_2 was degassed with N_2 and served as electrolyte during experiments. Two electrochemistry techniques, cyclic voltammetry and amperometry, were used to characterize the sensor response to glucose and lactate. D-glucose and lactic acid solutions were stored overnight at room temperature to allow equilibration of the α - and β -forms.

To characterize redox properties of vinylferrocene-containing hydrogel cyclic voltammetry was used with scan rates ranging from 10 to 50 mV/s. When performing amperometry experiments, enzyme-containing working electrode was poised at 0.4 V (vs. Ag/AgCl) (anodic peak potential of immobilized vinylferrocene) and exposed to aliquots of glucose or lactate. Analyte concentration in the electrochemical cell was adjusted in 1 to 2 mM increments. Analytes were added after the background current has stabilized. For sensor stability experiments, fabricated enzyme electrodes were stored at 4°C in 1x PBS between tests.

RESULTS AND DISCUSSION

In the present study, PEG hydrogel microfabrication was employed to integrate and package miniature Au electrodes with enzyme-carrying hydrogel structures. This novel fabrication approach allowed to deposit GOX- and LOX-carrying hydrogels on the adjacent members of an electrode array. Electrochemical testing was employed to characterize sensor response to glucose and lactate, and to demonstrate simultaneous response to both analytes from the same electrode array. The use of PEG hydrogel, a non-fouling and enzyme-friendly material, for construction of miniature enzyme electrodes opens future possibility for interfacing electrodes with living cells for dynamic monitoring of cell function.

Surface modification and fabrication of electrodes

An array of individually addressable Au electrodes (layout shown in Figure 1) consisted of 300 μm diameter electrodes connected to contact pads via 15 μm wide leads. An image of three electrodes is shown in Figure 2A. Upon patterning of Au electrodes, substrates were modified with acrylated silane to ensure covalent attachment of hydrogel structures to glass. We found that silanization of glass substrates containing unprotected Au patterns resulted in highly insulated and resistive electrodes. To resolve this problem, we did not remove the photoresist immediately after fabricating of the Au electrode arrays but used it as a protective layer during the silanization step. The photoresist layer was then removed after silane modification. Soluble

ferricyanide redox species were used to test differences in electron transfer after silane modification of unprotected vs. photoresist protected electrodes. As shown in cyclic voltammetry analysis presented in Figure 2B, the electrodes that were protected by photoresist during silanization were nearly as conductive as bare, unprocessed electrodes. In contrast, bare Au electrodes exposed to silane solution were effectively insulated by the self-assembled organic layer and could not be used for electrochemistry experiments (Figure 2B).

Fabrication of enzyme-carrying hydrogel microstructures on Au electrodes

Prior to enzyme immobilization, a layer of PEG hydrogel was micropatterned on the substrate so as to cover the leads while leaving open circular Au electrodes. This was necessary in order to decrease the background current and to enhance sensitivity of enzyme electrodes. As characterized by cyclic voltammetry with soluble ferricyanide (see Figure 2B), immobilizing pure PEG (not carrying redox species) on top of Au electrodes resulted in effective insulation of the electrodes. Combined with biocompatibility; insulating properties make PEG hydrogel an excellent bio-electrode packaging material.

As can be seen from Figure 1A and Figure 2A, an electrode array represents a complex pattern comprised of electrodes, leads, and contact pads. In order for array members to function as individual biosensors, enzymes must be placed exclusively onto specific electrodes without coating adjacent electrodes and/or interconnects. In this study, we employed PEG hydrogel photolithography method(30, 36) to deposit enzyme-carrying hydrogel microstructures onto desired members of an electrode array. Because PEG hydrogel patterning is analogous to traditional photoresist lithography, standard semiconductor tools, such as a mask aligner, could be employed to fabricate hydrogel microstructures in registration with microfabricated Au electrodes. Figure 2C shows two 300 μm diameter Au electrodes registered with 600 μm diameter PEG hydrogel elements.

Beyond depositing identical hydrogel microstructures on all electrodes, we wanted to highlight possibility of placing hydrogel structures of distinct composition on adjacent electrodes. To help visualize this, two different PEG precursor solutions were patterned on Au electrodes using a two mask process described diagrammatically in Figure 3A. The first PEG solution contained 50 μM Amplex Red and 0.1 mg/mL HRP while the second precursor solution was pure PEG. Figure 3B shows Amplex Red/HRP containing hydrogel structure fabricated on top of one Au electrode while the adjacent electrode remains bare. This image underscores our ability to spatially control placement of distinct hydrogel elements on a microfabricated surface. Exposure of this micropatterned surface to soluble 10 μM H_2O_2 initiated HRP-catalyzed breakdown of this molecule and conversion of non-fluorescent Amplex Red into a fluorescent intermediate resorufin.(36) This resulted in coloration of the hydrogel structure seen in Figure 3C. In the next fabrication step, pure PEG precursor solution was photopolymerized adjacent to the enzyme-carrying hydrogel. When exposed to H_2O_2 , HRP/Amplex Red-containing hydrogel element on the right changed color while pure PEG element remained colorless (see Figure 3C). The images in Figure 3(B,C) provide proof-of-concept demonstration that hydrogel microstructures of different composition can be fabricated on adjacent electrodes.

Charactering redox activity of hydrogel/Au electrodes

After fabricating hybrid hydrogel/Au electrodes in a manner analogous to that described in Figure 3, we proceeded to characterize response of these biosensors to analytes of interest: glucose and lactate. As discussed in the previous section, pure PEG hydrogel layer was found to be highly insulating, likely due to a small molecular weight PEG (MW 575) creating tight polymer mesh on top of an electrode. To improve conductivity, vinylferrocene was included into the prepolymer solution and became covalently incorporated into the PEG gel upon UV

cross-linking. Incorporation of redox sites into the gel was also expected to mediate electron transfer from oxidoreductase enzymes to the electrode surface. This enzyme “wiring” strategy first described by Heller and colleagues(37) eliminates the reliance of glucose and lactate sensors on diffusible species such oxygen (co-substrate) and hydrogen peroxide (product). The oxygen tension and/or hydrogen peroxide concentration in the body or in the vicinity of cultured cells may vary and may contribute to erroneous sensor reading. Oxygen independence of glucose and lactate detection is described in the next section of this paper, while the redox activity of the gels is discussed below.

The activity of the redox species within the hydrogel was characterized by cyclic voltammetry at different scan rates ranging from 10 – 50 mV/s in PBS. As shown in Figure 4A, electrodes covered with redox hydrogels had anodic and cathodic peaks at 400 and 340 mV respectively which is consistent with redox behavior of ferrocene.(38) The amplitude of anodic and cathodic peak did not change appreciably when testing the electrodes after 2 weeks of incubation in PBS (data not shown), pointing to retention of the redox species in the gel. Importantly, Figure 4A also shows that peak splitting was unchanged as a function of scan rate, reverse-to-forward peak current ratio was close to unity and that the peak current increased proportionally to the scan rate (see Figure 4B). This suggests fast and reversible redox processes occurring in the PEG hydrogel-modified electrodes.

Importantly, electrodes within the same array worked independently. Figure 4C shows cyclic voltammograms from adjacent electrodes containing conductive hydrogel microstructures. As seen from these data, the current of two electrodes connected together was twice as high as that of individual electrodes. Similar testing was carried out before analyte detection to ensure that no short circuiting of the electrodes occurred after fabrication.

Detecting glucose and lactate with enzyme-carrying hydrogel/Au electrode arrays

Amperometry was used to analyze the biosensor response to glucose and lactate of varying concentration. In these experiments, individual members of hydrogel/Au electrode array were poised at 0.4V vs. Ag/AgCl reference electrode and the changes in current in response to aliquotal addition of analyte were recorded. Figure 5A shows the current-time relationship of the hybrid hydrogel/electrode arrays to 2 mM glucose aliquots added into PBS solution (pH = 7.0). As seen from these data, electrode response to glucose achieved saturation after 60 sec; therefore, we chose this time interval between successive additions of glucose aliquots. The glucose calibration curve shown in Figure 5B was constructed by averaging the amperometric responses from a five-electrode array. The response was linear with a R^2 of 0.99 and a sensitivity of $0.9 \mu\text{A cm}^{-2} \text{mM}^{-1}$ in the range from 0 to 20 mM. The standard deviation between members of the same electrode array was ~6 % while the standard deviation between devices was ~8% (n=5). This underscores intra- and inter-device reproducibility of glucose response. The response of LOX-containing hydrogel/Au electrodes to lactate was characterized by amperometry in a manner similar to that described for glucose sensor above. Figure 5(C,D) shows biosensor response to aliquotal addition of 1 mM lactate into PBS solution (pH = 7) and the resultant calibration curve that was constructed by averaging response from a five-electrode array. The lactate response was linear with a R^2 of 0.98 and a sensitivity of $1.1 \mu\text{A cm}^{-2} \text{mM}^{-1}$ in the range from 0 to 10 μM . The electrode responded rapidly to the addition of glucose and lactate, reaching 90% of the signal within 70 sec.

While the sensitivity of the hydrogel-based electrodes described here is comparable or better than some of the previous results reported by us and others,(22–24,39) there have been other studies demonstrating more sensitive glucose electrodes.(2,40) The sensitivity of electrodes described here may be enhanced in the future by altering gel composition and/or decreasing the electrode size to approach microelectrode dimensions.

The PEG photolithography afforded us the possibility to immobilize hydrogel structures of distinct composition onto members of the same electrode array (see Figure 3 for example). This strategy was used to construct electrode arrays where individual Au electrodes were functionalized with GOX- and LOX-carrying hydrogel microstructures. The ability of this biosensor array to detect two analytes, glucose and lactate, was then characterized by amperometry in a manner similar to that described for single analyte detection. Figure 6 demonstrates an experiment where adjacent electrodes containing GOX- and LOX-carrying hydrogels were connected together to a potentiostat and challenged first with lactic acid and then glucose. As seen from these data, the sensor first responded to lactate with sensitivity comparable to that demonstrated for a single lactate sensitive in Figure 5C. Subsequent exposure to glucose resulted in response equivalent to a single glucose sensitive electrode characterized in Figure 5A. These experiments demonstrate that both types of biosensors were present in the same electrode array.

Stability and oxygen independence of hydrogel-based enzyme electrodes

It is very important that an enzyme-based biosensor is stable and is unaffected by changes in the surrounding environment. The stability of the biosensor was also investigated by challenging GOX-containing hydrogel/Au electrodes to 2 mM glucose daily for 15 days. As shown in Figure 7A glucose biosensor remained stable, retaining ~90% of its original response over this period of time.

Another important parameter tested was oxygen dependence of our biosensors. Oxygen is a co-substrate consumed in the enzymatic breakdown of both glucose and lactate, and can be used for detection of both analytes. However, oxygen tension may vary *in vivo* or in proximity of metabolically active living cells in a culture dish. Heller and colleagues proposed to complex or “wire” enzymes to redox centers to enable direct electron transfer from the enzyme to the electrode via the redox sites, thus eliminating sensor dependence on oxygen.^(2,37) In the present study, vinyl-terminated ferrocene was included into a prepolymer solution and became covalently incorporated into gel microstructures after photopatterning. As described in Figure 4 above, the redox sites were stably immobilized and electrochemically active within the hydrogel. To investigate sensitivity of our biosensors to oxygen, GOX-containing hydrogel/Au electrodes were challenged with glucose dissolved in either PBS containing ambient oxygen (21%) or PBS solution that was deoxygenated by N₂ purging (0% oxygen as measured by commercial Clark-type electrode). Figure 6B shows signal from an enzyme electrode response to glucose dissolved in either oxygen-containing or oxygen-free electrolyte. A slightly lower (~8%) glucose signal was observed in the case of electrolyte containing oxygen. This difference in signal is minor - Heller et al. reported comparable differences in their “wired” electrodes. (4) While hydrogen peroxide oxidizes at much higher potentials (~0.7V vs. Ag/AgCl) minimal oxidation may be present in our experiments (performed at 0.4V vs. Ag/AgCl) and may contribute to a slightly higher signal in the presence of oxygen. This problem may be addressed in the future by selecting a redox molecule possessing lower formal potential.

CONCLUSIONS

Our paper describes the use of biomaterial microfabrication technique – PEG hydrogel photolithography – as a novel strategy for integrating miniature Au electrodes and enzyme-carrying hydrogel microstructures. PEG photolithography mimicked multilayer, multi-mask processes employed in semiconductor device fabrication and allowed to immobilize GOX- and LOX-carrying hydrogels onto microfabricated Au electrode arrays. The biosensors were characterized electrochemically and exhibited a linear detection range of 20 mM for glucose and 10 mM for lactate. The sensitivity of the biosensors was $0.9 \mu\text{A cm}^{-2} \text{mM}^{-1}$ for glucose and $1.1 \mu\text{A cm}^{-2} \text{mM}^{-1}$ for lactate. Significantly, both glucose and lactate could be detected

from the same electrode array. The microfabricated biosensors were highly reproducible with a standard deviation of 6 % in glucose response between each array members and were stable after two weeks of testing. Inclusion of redox sites into the hydrogel ensured that enzyme electrodes were insensitive to oxygen tension. Besides providing an excellent matrix for enzyme entrapment, PEG hydrogels are non-fouling and have been used extensively by our group and others for micropatterning of living cells.(41–44) In addition, we recently described sensing hydrogel microstructures that may be used to both sequester cells and to detect cell-secreted metabolites with fluorescence.(33) The present paper provides a blueprint for fabricating and packaging miniature enzyme-based electrodes using a biocompatible, non-fouling and cell-friendly polymer. In the future, dual functionality of PEG hydrogel microstructures as biosensors and cell culture chambers will be used for electrochemical detection of metabolite fluxes in the immediate vicinity of living cells.

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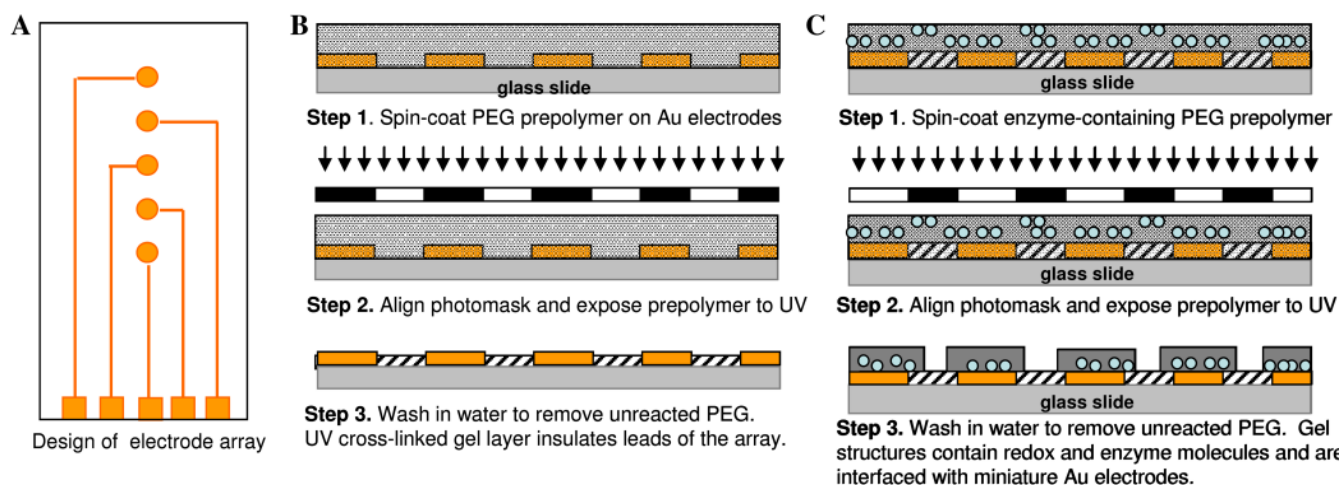


Figure 1.

(A) Layout of an electrode array consisting of five 300 μm diameter Au electrodes, 15 μm wide leads and 4 mm^2 contact pads. (B) Micro patterning PEG hydrogel to insulate the leads. This hydrogel layer does not have redox polymer or enzyme molecules. (C) Micro patterning enzyme-carrying hydrogel microstructures in alignment with microfabricated Au electrodes. This process can be repeated with multiple masks and different prepolymer formulations to immobilize distinct enzyme-carrying gel structures on different electrodes of the array.

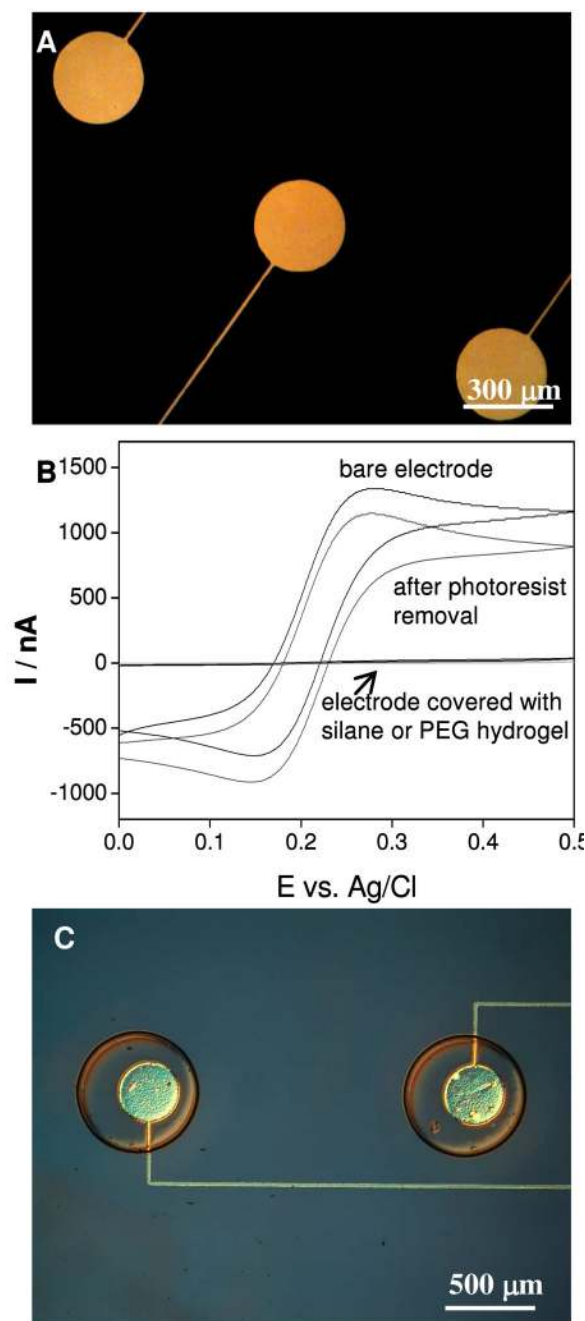


Figure 2.

(A) A portion of five electrode array with 300 μm diameter Au electrodes and 15 μm diameter leads. Contact pads are not shown in this image. (B) Cyclic voltammetry characterization of bare vs. photoresist protected electrodes after silane modification. Ferricyanide was used as diffusible redox species to test the electron transfer. The scan rate was 20 mV/sec Ag/AgCl reference and Pt counter electrodes were used. Electrodes modified with silane or coated with hydrogel were similarly resistive and CV curves for these two conditions overlap. Insulating PEG hydrogel coating did not contain redox polymer. (C) Hydrogel microstructures (600 μm diameter) fabricated on top of the 300 μm Au electrodes. The hydrogel structures were made

larger than electrodes to ensure attachment of gel to the glass substrate modified with acrylated silane.

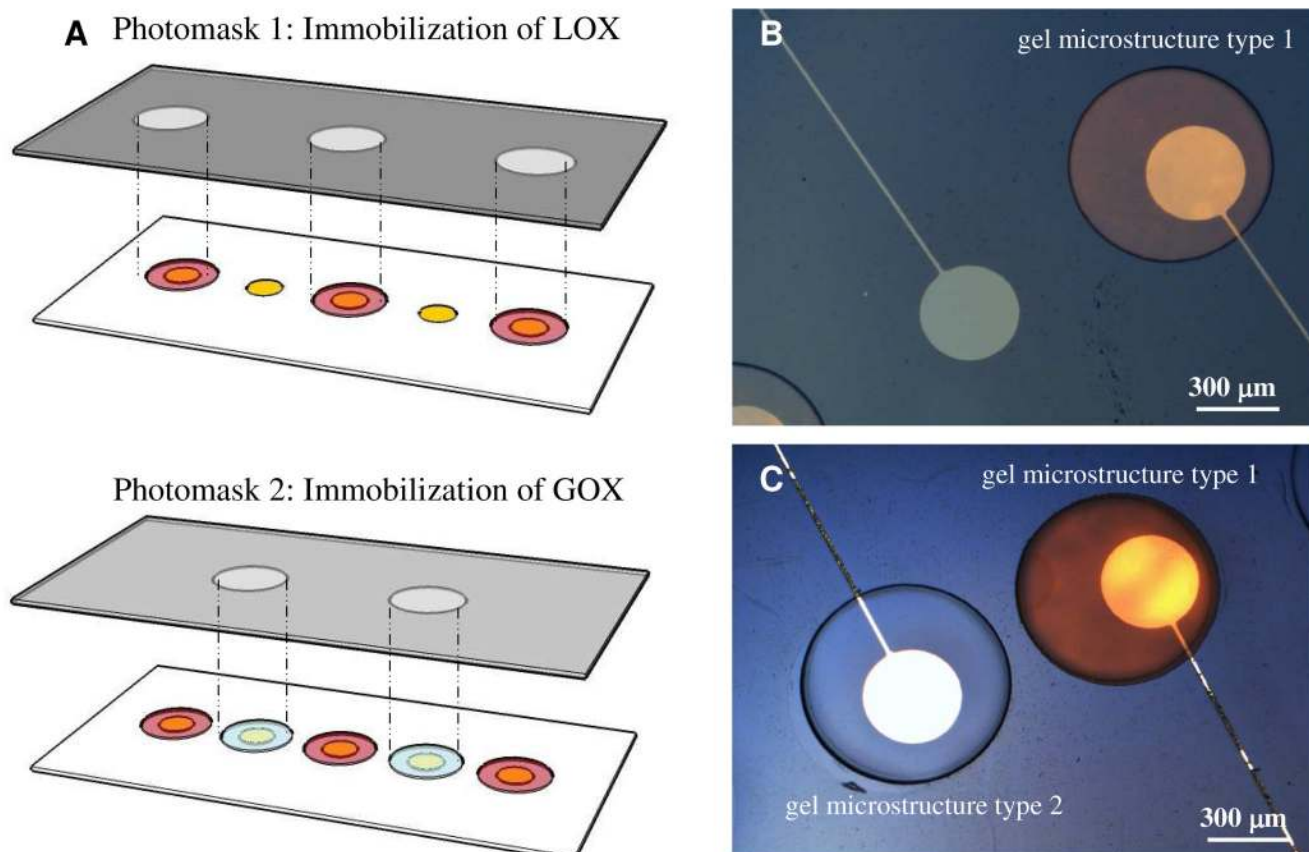


Figure 3.

(A) A prepolymer formulations containing either GOX or LOX was spun onto the surface and exposed through a photomask. Subsequently, the second prepolymer formulation was spin-coated on the surface and registered with Au electrodes that remained unmodified. These sequential two mask process sequential two mask process resulted in fabrication of glucose- and lactate-sensitive electrodes in the same array. (B–C) Alignment and deposition of hydrogel microstructures of varying content on microfabricated Au electrodes. Hydrogel type 1 contains peroxidase/Amplex Red and becomes fluorescent when challenged with 5 mM H_2O_2 whereas hydrogel type 2 deposited on an adjacent electrode does not have these reporter molecules and remains insensitive to analyte.

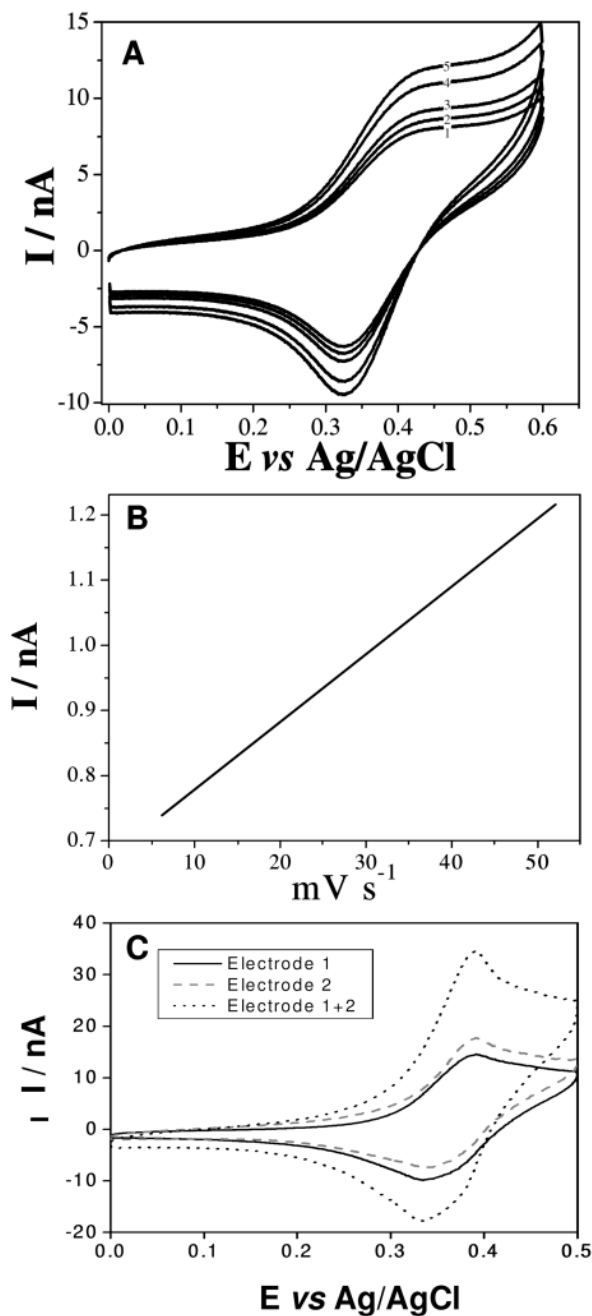


Figure 4. (A) Characterization of redox activity of vinylferrocene-containing hydrogel electrodes using cyclic voltammetry. Scan rate was varied from 10 mV/s to 100 mV/s. Ag/AgCl reference and Pt counter electrodes were used. (B) Linear relationship of anodic peak current vs. scan rate suggests fast and reversible kinetics. (C) Demonstration that adjacent hydrogel electrodes were individually addressable. Comparison of redox activity of single hydrogel/Au electrode vs. two electrodes connected together.

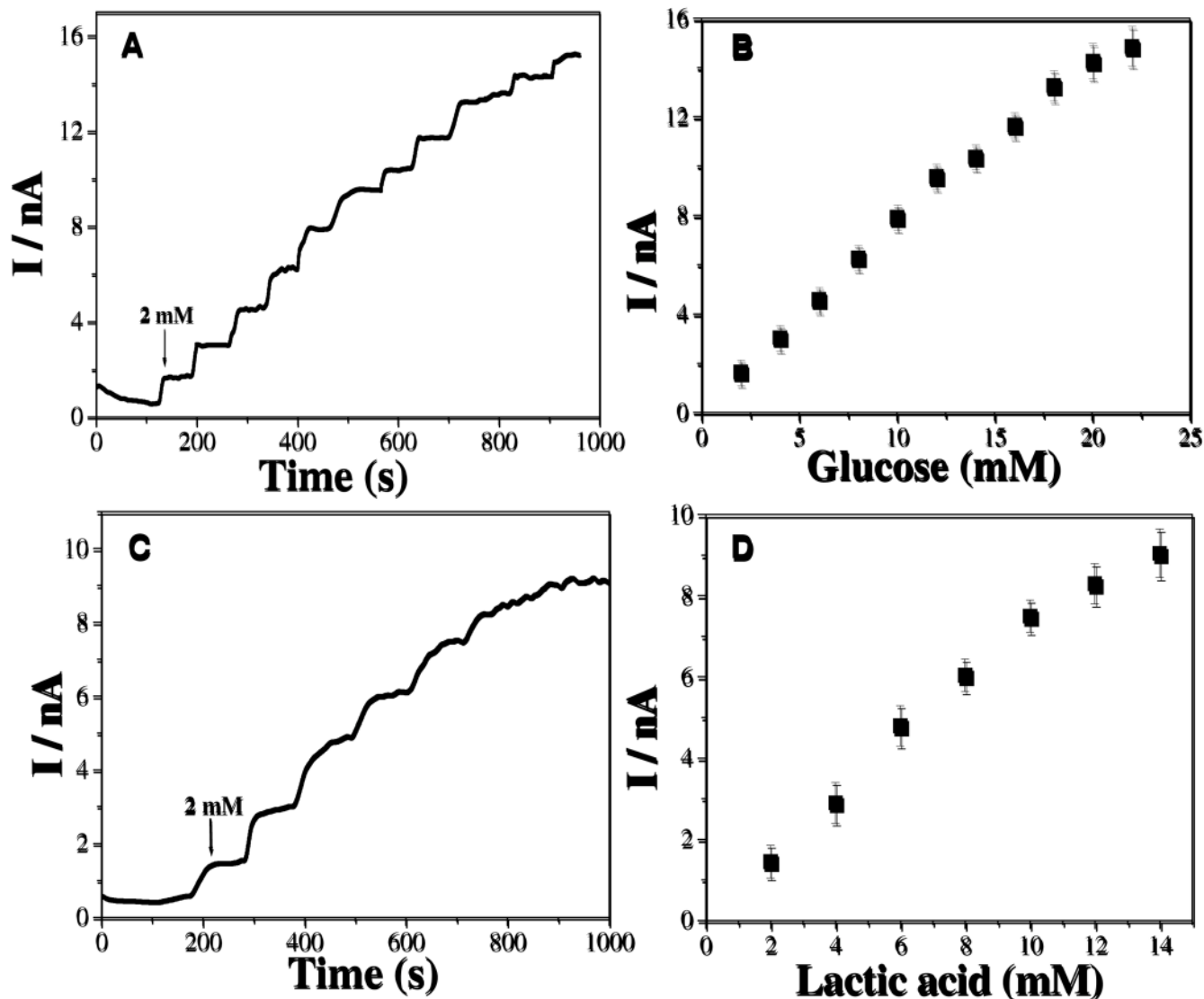


Figure 5.

(A) Amperometric response of miniature enzyme electrodes to glucose. Microfabricated GOX-containing hydrogel/Au electrodes were poised at 0.4 mV vs. Ag/AgCl reference electrode and were challenged with glucose in 2 mM increments. A current vs. time response was recorded and averaged for five different devices ($n=5$). (B) The calibration curve of GOX-based electrodes for 0 to 20 mM glucose range. (C) Amperometric response of LOX-containing hydrogel/Au electrodes to 2 mM aliquots of lactic acid. Conditions for testing the electrodes were identical to those discussed for glucose. (D) The calibration curve of lactate electrode showing response in the 0 to 10 mM range of analyte.

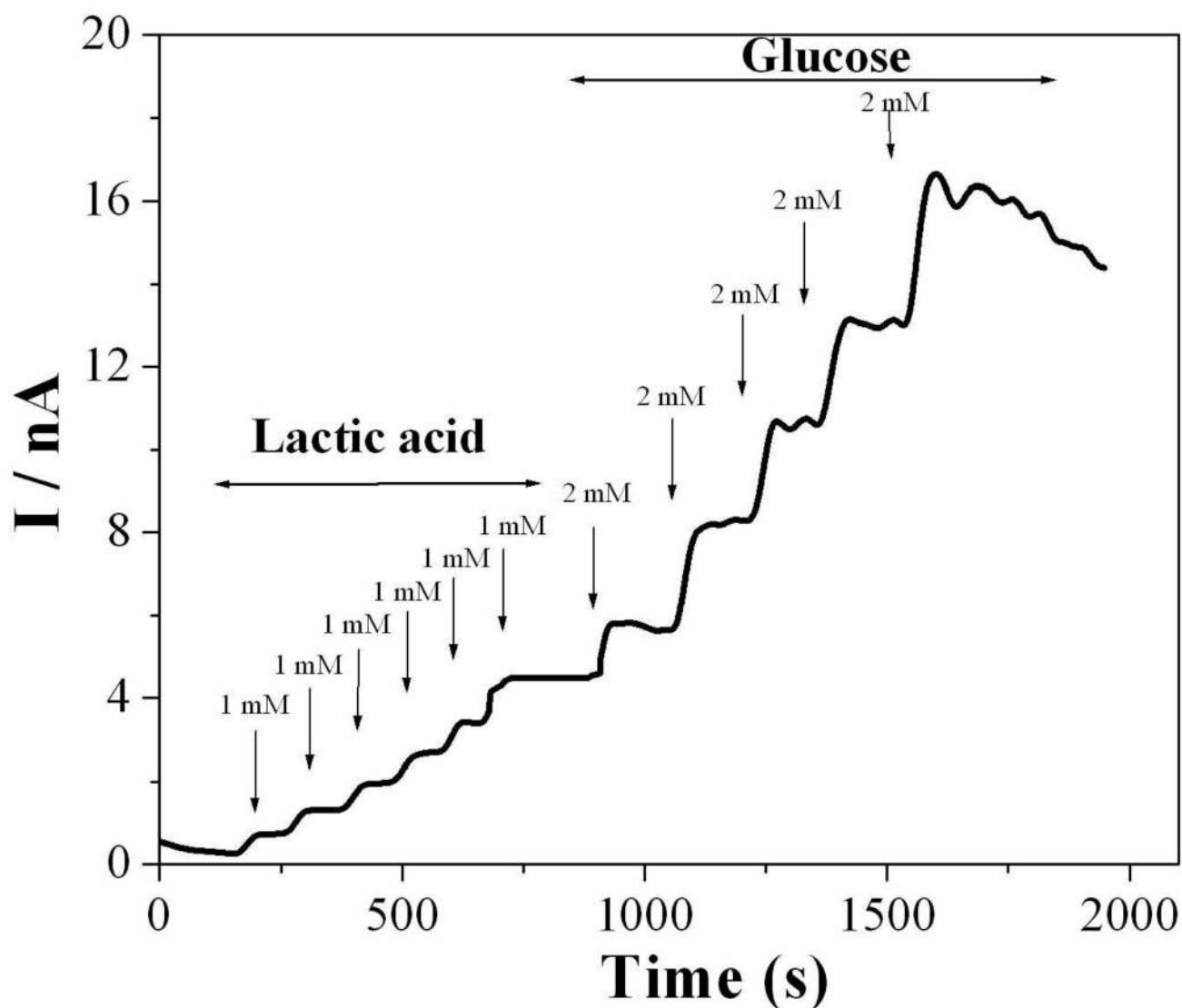


Figure 6. Connecting together two members of an electrode array, one GOX-containing hydrogel/Au electrode another LOX-carrying hydrogel/Au electrode. This electrode pair was challenged with lactic acid followed by glucose. Signals were recorder by amperometry by posing electrodes at 0.4 V vs. Ag/AgCl reference. The response of an electrode pair to each analyte was comparable to that of individual glucose and lactate electrodes. This result shows that glucose and lactate can be detected in the same array of hydrogel/Au electrodes.

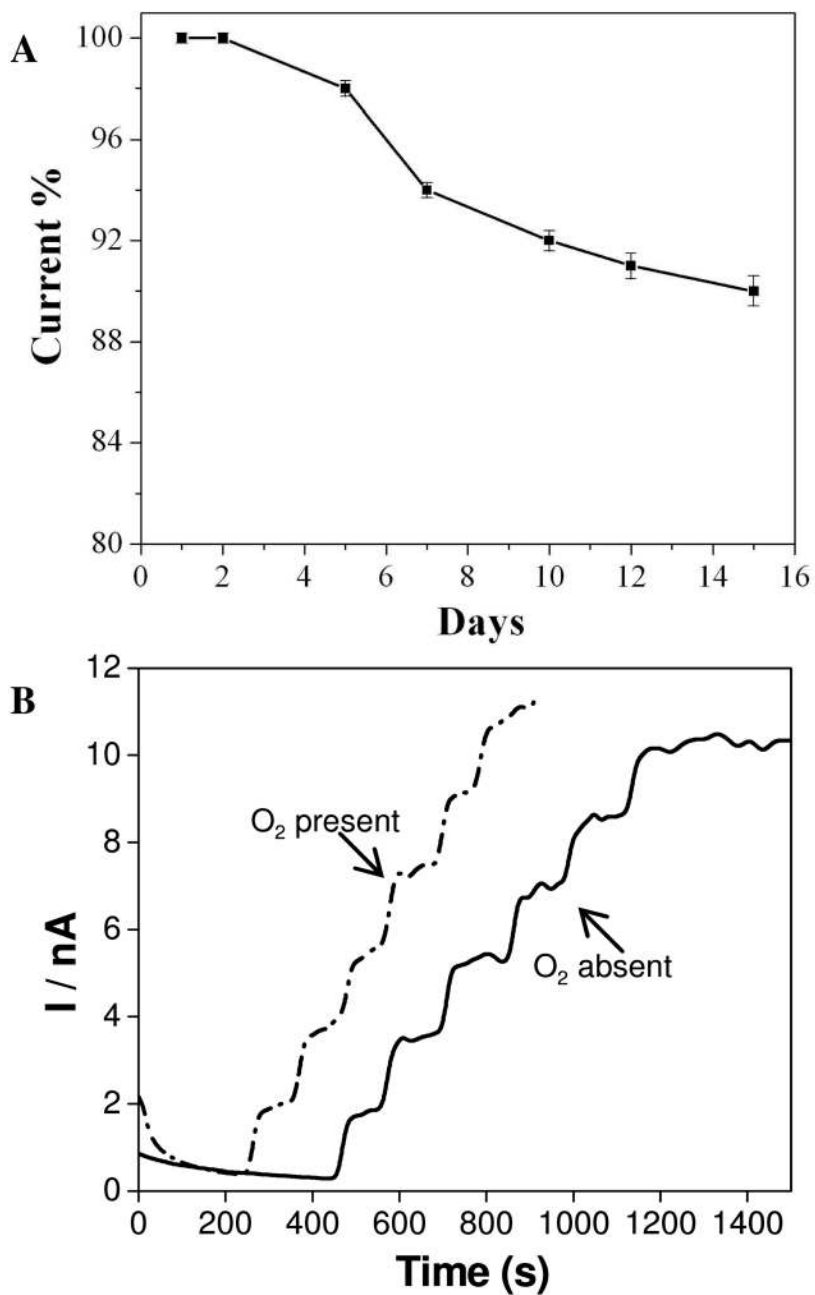


Figure 7. (A) Testing stability of glucose electrodes. Electrodes were stored at 4°C in PBS and were tested daily by exposure to 6 mM glucose. Response to glucose was recorded as current vs. time plot for five different devices ($n=5$). (B) Response of GOX-carrying hydrogel/Au electrodes to 2 mM aliquots of glucose dissolved in oxygen-containing (21% oxygen) and oxygen-free PBS. Commercial oxygen electrode was used to verify oxygen tension of electrolyte solution. Electrode was poised at 0.4V vs. Ag/AgCl reference.